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BIOCHEMICAL BASIS OF VIRULENCE IN EPIDEMIC TYPHUS

Annual Progress Report

Herbert H. Winkler, Ph.D.

August 1979

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College of Medicine
Mobile, Alabama 36688

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Rickettsia prowazeki were assessed for their in vitro susceptibility to phagocytosis by rabbit polymorphonuclear leukocytes (PMN). ATP labeling was used to quantitatively determine phagocytosis and adsorption. Rickettsiae were less susceptible to phagocytosis then were Escherichia coli and Neisseria gonorrhoeae. Although rickettsiae were similar to E. coli in susceptibility to superoxide and activated halide, few phagocytized rickettsiae were inactivated 45 min after being ingested by PMN and some rickettsiae were

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observed free in the PMN cytoplasm. At low rickettsia: PMN multiplicities, phagocytosis increased as a linear function of time, but at high multiplicities (MOI=50) rickettsiae were phagocytized during only the first 10 min of incubation. PMN were damaged in the presence of high rickettsial multiplicities such that they released lactate dehydrogenase into the medium and lost the ability to phagocytize both rickettsiae and E. coli. This rickettsial leukotoxic activity was associated with phospholipase activity which was similar to the phospholipase activity associated with rickettsial hemolysis, and the amount of leukotoxic activity in a given rickettsial sample correlated with the relative hemolytic activity of that sample. The rickettsial leukotoxin was probably not a soluble product, was not inhibited in the absence of phagocytosis, and was inhibited by inactivation of the rickettsiae, incubation at 4°C or the addition of NaF to the incubation mixture. Unlike the hemolytic process, however, rickettsiae did not perceptably adhere to PMN as part of the lytic process.

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SUMMARY

The purpose of the work is to elucidate the biochemical basis of virulence in epidemic typhus. The basic method at this stage of the project is a comparison of the E strain and Breinl strain (avirulent and virulent, respectively) of Rickettsia prowazeki and there interaction with the host defense system.

To date (six months) macrophage cell lines, peritoneal macrophages and polymorphonuclear leucocytes have been investigated as to their interaction with the E strain. Because of the short time only the latter study, PMN-rickettsial interactions are complete enough to be presented in a formal annual report.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal, Resources, National Academy of Sciences-National Research Council.

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INTRODUCTION

When typhus rickettsiae are introduced into a nonimmune host there is little host response until rickettsiae burst from infected endothelial cells (22,25). This burst is rapidly followed by local accumulation of polymorphonuclear leukocytes (PMN) with some PMN containing ingested rickettsiae. Using the skin window technique, Wisseman et al. (25) reported that in both immune and nonimmune hosts there was a marked PMN response to typhus rickettsiae during the first 24 h with increased accumulation of macrophages thereafter. Both PMN response and degree of phagocytosis were greater in immune hosts but these parameters could not be quantified. Opsonizing antibody appears in previously nonimmune hosts about 2 weeks after the onset of infection with either virulent or attenuated typhus rickettsiae.

Wisseman et al. (23) examined interactions between killed typhus rickettsiae (R. mooseri and R. prowazeki) and PMN in vitro. At high rickettsia: PMN multiplicities (125:1), phagocytosis of R. mooseri increased as a function of time for about 15 min and then leveled off, with little or no subsequent phagocytosis. Less than 1% of the rickettsial suspension was phagocytized under these conditions, but phagocytosis was greatly stimulated in the presence of specific anti-rickettsial antiserum. Similar low phagocytic values were obtained using live or dead rickettsiae and various virulent and attenuated rickettsial strains. It was felt, however, that this level of phagocytosis was not unusually low because "rough" isolates of Brucella abortis and Pasteurella multocida were phagocytized to a similar low extent. Furthermore, consistent with skin

window observations, rickettsiae were chemotactic for PMN *in vitro* (22).

We examined *in vitro* interactions between rabbit PMN and attenuated typhus rickettsiae under a variety of conditions. PMN were assessed for their relative ability to phagocytize and kill *R. prowazeki*, Madrid E strain using *Escherichia coli* and T4 gonococci for comparison. Because Wisseman *et al.* demonstrated that phagocytosis of *R. mooseri* did not continue beyond the first 15 min of incubation, we also assayed for possible toxic effects of rickettsiae to PMN.

MATERIALS AND METHODS

Rickettsial preparation and growth

R. prowazeki, Madrid E strain was propagated in 6-day, embryonated, antibiotic-free chicken eggs by inoculation from a seed pool (yolk sac passage no. 274). Rickettsial suspensions were prepared from heavily infected yolk sacs by a modification of the methods of Bovarnick and Snyder (5) and Wisseman *et al.* (24) as previously described (20). Only fresh, unfrozen rickettsiae were used.

The diluent for the rickettsial inoculum and rickettsial suspension in the purification procedure was a sucrose-phosphate-glutamate (SPG) solution originally described by Bovarnick *et al.* (4). The diluent for sheep erythrocytes was SPGMg (SPG containing 0.01 M $MgCl_2$). The diluent for PMN - association assays was a modified Hanks solution containing 0.01% bovine serum albumin, 0.1% glucose (HBG; ref. 10), 0.01 M $MgCl_2$ and L-glutamic acid (HBGMgGlut).

Rickettsiae were enumerated by a modification of the method of Silberman and Fiset (17); rickettsia-like bodies (RLB) were enumerated

directly rather than by photography.

Radioactively labeled rickettsiae were prepared by incubation of rickettsiae with [α-^{32}P] ATP as previously described (21).

Radioactively-labeled Escherichia coli were prepared by incubating an aliquot of an overnight culture of E. coli, strain E15, for 3 h at 37°C in a minimal growth medium (Medium A;6) containing 2% D-glucose and 0.1 µCi/ml ^{3}H-glucose. The bacteria were then washed twice and resuspended in HBGMgGlut.

Gonococci were labeled by spreading 3 µCi ^{3}H-glucose on the surface of a GC agar plate; the plate was then inoculated with type (T) 4 gonococci which were carefully selected with the assistance of a dissecting microscope (8). Gonococci were harvested by washing them off the agar surface with warm (37°C) HBGMgGlut, then washed twice and resuspended in HBGMgGlut.

Hemolytic assay

The hemolysis tests were modifications of the method of Snyder et al. (18, as previously described (14,15).

Phagocytosis

PMN-rich leukocytic exudates were induced in rabbits by the intraperitoneal injection, 24 h earlier, of 250 ml of 0.85% NaCl containing 1% shellfish glycogen. Sterile exudates were collected in HBG, washed five times, resuspended in HBG and counted in a hemocytometer. All exudates used for phagocytic studies consisted of >90% PMN.

PMN were tested for phagocytosis of bacteria in the "dilute" phagocytic test system (19). Briefly, 1.25 x 10^{8} PMN were concentrated by

centrifugation at 230 x g for 5 min at 4°C in a 13 by 100 mm screw-capped tube, mixed with an appropriate number of bacteria and the total volume was adjusted to 500 µl by the addition of HBGMgGlut or serum. The suspensions were then tumbled end-over-end at 12 rpm at 37°C for the indicated time. To distinguish intracellular from external, adherent rickettsiae, 1mM unlabeled ATP was added to samples 1 min before the end of the incubation. Previous studies (21) showed that the addition of excess unlabeled ATP results in a complete loss of label from rickettsiae within 1 min. Efflux of ATP was identical in SPGMg and in HBGMgGlut. After incubation, 1 ml HBGMgGlut was added and PMN were pelleted as before. Supernatant aliquots were removed for determination of the concentration of cell-free lactate dehydrogenase (LDH) and for assessment of loss of radioactivity from rickettsiae. PMN were washed twice in 4 ml HBGMgGlut and lysed by resuspension in 2 ml 5% Triton X-100. 0.5 ml of each sample was then mixed with 5 ml of TT21 and 1 ml water and the radioactivity was determined.

Treatments

Rickettsiae were inactivated by exposure to UV radiation (8-W germicidal lamp) for 20 min at 0°C and a distance of 12 cm or by treatment for 15 min at 37°C with 1 mM N-ethylmaleimide (NEM); unreacted NEM was neutralized by the addition of 10 mM 2:mercaptoethanol. The ability of rickettsiae to lyse sheep erythrocytes (14,15,18) was used as an indicator of rickettsial activity.

Neutrophil phagocytic activity was inhibited by incubation of PMN's with 10 µg/ml cytochalasin B or 40 mM NaF at 37°C for 15 min before

addition of and during incubation with bacteria or by incubation of neutrophils and bacteria at 4°C.

In vitro bactericidal assay

Rickettsial killing by products of the purine-xanthine oxidase system was determined by a modification of the system of Babior et al. (2). 25 µl of a rickettsial suspension (about 10^9 rickettsiae) was incubated in a 250 µl system containing 0.1 to 1.0 U/ml XO, 25 l of 50 mM purine and the balance SPGMg. Other additions, in selected cases, included superoxide dismutase (SOD: 1 mg/ml), catalase (0.25 mg/ml), allopurinol (0.5 mM), lactoperoxidase (1 mg/ml) and KI (0.1 mM). The production of superoxide by xanthine oxidase was measured by determining SOD-inhibitable cytochrome c reduction (11). Rickettsial inactivation was measured by spectrophotrometric assessment of rickettsial hemolytic activity.

To assess the in vitro rickettsiacidal activity of rabbit PMN, rickettsiae and PMN were incubated with tumbling at a multiplicity of 10 for 20 min at 37°C. A portion was then removed and the PMN were broken by blending in a Sorvall Superspeed Blender for 60s (two 30s bursts) at 4°C. Extracellular rickettsiae were then separated from PMN-associated rickettsiae by centrifugation, PMN were washed twice in HBGMgGlut and the washed PMN were suspended to 0.5 ml in HBGMgGlut, placed in a 13 x 100 mM tube and incubated at 37°C. At various times, a portion was removed and blended as before to release intracellular rickettsiae. To determine relative rickettsial activity, each blended aliquot and the first wash supernatant were assessed for hemolytic activity as previously described except that, in order to increase the sensitivity of the assay, hemolysis

was allowed to proceed at 34°C for 3 h. Controls included blended and nonblended suspensions of rickettsiae plus PMN or rickettsiae alone and blended rickettsial suspensions which were incubated as above but without PMN.

HMP Shunt Activity HMP shunt activity was monitored by determination of PMN $^{14}CO_2$ evolution from 1-^{14}C-glucose. Briefly, PMN and rickettsiae were incubated in a 10 ml sealed vial at 34°C at a multiplicity of 10 in the presence of 1-^{14}C-glucose (0.15 μCi/ml). When indicated, 0.4 ml ethanolamine was carefully injected into a plastic cup suspended above the reaction suspension to trap evolved $^{14}CO_2$. The surface area and, thus, the efficiency of the CO_2 trap was increased by placing a 1 in. by 3/4 in. piece of fluted filter paper (Whatman No. 1) in the CO_2 trap before sealing the vial. After addition of the ethanolamine, 0.4 ml 2N HCl was injected into the phagocytic suspension to inactivate the suspension and to drive off CO_2. CO_2 collection was allowed to proceed for 60 min at room temperature and then aliquots of PMN suspensions and the entire contents of each trap were placed in scintillation vials along with 10 ml TT21 and radioactivity was determined in a Beckman liquid scintillation counter.

Lipid methodology

Suspensions of rickettsiae and PMN were extracted by the method of Bligh and Dyer (3). To each 1 ml of sample was added 0.3 ml EDTA (100 mM, pH 4.3), 1.6 ml chloroform and 3.2 ml methanol. The monophasic samples were then gassed with N_2 and extracted overnight at 4°C. The lipids were separated from water soluble material by adding 1.6 ml each of chloroform and water per ml of original suspension and centrifuging to separate the

phases. The chloroform layer was evaporated to dryness in a Savant speed-vac concentrator, dissolved in chloroform:methanol (2:1) and stored at -20°C under nitrogen.

One-dimensional seperation of neutral lipids was performed with silica gel (SG-G) thin layer plates using a solvent consisting of petroleum ether:diethyl ether:acetic acid (80:20:1). Two-dimensional separation of phospholipids on silica gel (SG-60) thin layer plates was performed using solvent I consisting of chloroform:metnanol:NH_4OH (65:25:5) and solvent II consisting of chloroform:acetone:methanol:glacial acetic acid:water (6:8:2:2:1).

RESULTS

Phagocytosis of rickettsiae

Because rickettsiae and PMN are susceptable to changes in media composition, several media were examined for their ability to support both rickettsial and PMN function. SPGMg, a standard rickettsial suspension medium was not suitable for phagocytic studies because of its high viscosity. As Table 1 shows, rickettsiae were unable to lyse erythrocytes normally after incubation in HBG, a medium often used to sustain PMN phagocytic activity (10). The addition of glutamate, the primary rickettsial energy source, to HBG partially restored hemolytic activity, and rickettsiae suspended in HBG with Mg and glutamate added (HBGMgGlut) were similar to control rickettsiae in their ability to lyse erythrocytes. Furthermore, α-^{32}P-ATP labelled rickettsiae retained their label similarly in HBGMgGlut and SPGMg, and when excess unlabelled ATP was added to suspensions of such rickettsiae, the exit of labelled ATP was identical in

both media (Fig 1).

PMN were assessed for their ability to phagocytize E. coli in the presence of HBG or HBGMgGlut (Table 1). Phagocytosis of E. coli was slightly stimulated in HBGMgGlut. Because rickettsial and PMN functions in HBGMgGlut were similar to control values, HBGMgGlut was used for studies on interactions between rickettsiae and PMN.

Phagocytosis of rickettsiae increased as a function of rickettsia:PMN multiplicity (Fig 2), but few additional rickettsiae were phagocytized in 20 min beyond the values obtained at a multiplicity of 10. In contrast, T4 gonococci and E. coli were rapidly phagocytized with phagocytosis increasing linearly up to a multiplicity of at least 50.

Thus, at a multiplicity of 50, 8 times as many N. gonorrhoeae and 12 times as many E. coli as R. prowazeki were phagocytized in 20 min. Yet at a multiplicity of 5, the respective relative difference in phagocytosis were only 2-fold and 3-fold. This suggested not only that rickettsiae were less susceptible to phagocytosis than were T4 gonococci and E. coli, but that large numbers of rickettsiae might be toxic to rabbit PMN.

At a bacteria:PMN ratio of 1 (Fig 3B) phagocytosis of ricketsiae increased linearly with time, but at a very slow rate relative to that of E. coli. At a multiplicity of 50, however, (Fig 3A) phagocytosis of rickettsiae reached a maximum after 10 min and then slowly decreased thereafter. The initial rate of phagocytosis at the higher multiplicity was slower than that of E. coli but similar to that of T4 gonococci. Phagocytosis of NEM-inactivated rickettsiae at a multiplicity of 50, which was slower than that of E. coli and gonococci, increased linearly with

time for 30 min at 37°C without evidence of the "plateau" seen with native rickettsiae. Rickettsiae did not adhere to PMN; at each time point, almost all of the PMN-associated rickettsiae were inaccessible to the chase and, hence, intracellular. Phagocytosis of rickettsiae was inhibited by the presence, in the incubation medium, of 10 µg/ml cytochalasin B or 40 mM NaF or by incubating rickettsiae and PMN at 4°C (Table 2). Phagocytosis was greatly stimulated by the addition of 10% antirickettsial antiserum to the incubation mixture; normal rabbit serum had no effect.

Effects of rickettsiae on PMN

Because native but not inactivated rickettsiae at high multiplicities were phagocytized during only the first 10 min of incubation, PMN were examined for evidence of loss of function after incubation with rickettsiae. Table 3 shows that the ability of PMN previously incubated with high multiplicities of native but not inactivated rickettsiae to ingest E. coli was inhibited by almost 70%. Furthermore these PMN lost 30% of their lactate dehydrogenase (LDH) into the extracellular space. This indicates that native but not inactive rickettsiae damaged PMN sufficiently to cause both release of PMN cytoplasmic enzymes and significant inhibition of PMN phagocytic function. Subsequent experiments were performed using LDH release as an indicator of PMN damage.

LDH release from PMN increased linearly with time at 37°C when 50 native rickettsiae per PMN were present in the incubation mixture (Fig 4). PMN did not release LDH in the presence of inactivated rickettsiae. LDH release increased as a function of rickettsiae: PMN multiplicity, but this increase was not linear (Fig 5). Instead, LDH release was more closely

related to the hemolytic activity of each sample (r=0.93). This indicated that rickettsial energy was required for manifestation of the rickettsial leukotoxic activity and suggested that the rickettsial hemolytic system might be responsible for the PMN damage.

Consistent with this hypothesis was the observation that although cytochalasin B inhibited phagocytosis, it did not inhibit LDH release (Table 4). Thus, PMN could be damaged "from without" in the absence of significant phagocytosis. Incubation at 4°C, which inhibits phagocytosis and rickettsial hemolysis (14,15), also blocked release of LDH from PMN. When 10% as many rickettsiae were present but phagocytosis was stimulated by the presence of anti-rickettsial antibody such that the total number of rickettsiae ingested was nearly that found with a multiplicity of 50, LDH release was not elevated above the values normally obtained at a multiplicity of 5 in the absence of immune serum. Stimulation of phagocytosis of rickettsiae at a multiplicity of 50, however, resulted in a small decrease in the amount of LDH released. Thus, LDH release was not due simply to the ingestion of large numbers of rickettsiae.

Rickettsial hemolysis is closely associated with phospholipase activity (Winkler, personal communication); as hemolysis progresses there is a concomitant formation of lysophosphatides and free fatty acids. Treatments which block hemolysis also block phospholipase activity. We incubated rickettsiae with PMN under various conditions and assessed the formation of lysophosphatides and free fatty acids. Incubation of PMN with native but not NEM inactivated rickettsiae resulted in the formation of lysophosphatidylethenolamine (LPE) and free fatty acids (FFA). FFA

formation was confirmed by gas-liquid chromatography. LPE and FAA formation was normal in the presence of cytochalasin B, but was blocked by the addition of NaF, which also inhibited hemolysis. In order to eliminate the possibility that NaF effect was due to inhibition of phagocytosis, NaF incubations were performed in the presence of 10 µg/ml cytochalasin B.

Intraleukocytic killing of rickettsiae

Rickettsiae were assessed for susceptibility to intraleukocytic defense mechanisms using the purine-xanthine oxidase (XO) system (2). Rickettsial killing was monitered by measurement of loss of rickettsial hemolytic activity. Hemolysis is dependent on rickettsial metabolic activity (probably proton motive force); metabolically poisoned rickettsiae will not lyse erythrocytes (14,15). Fig 6 shows that killing of rickettsiae and E. coli, which was a linear function of time, was identical when the in vitro bactericidal system contained 0.5 U/ml XO. After 60 min incubation, viable rickettsiae and E. coli titers were both reduced by 1.3 $\log_{10}$. Killing was also a linear function of XO concentration between 0.25 and 1.0 U/ml XO (Fig 7). Although the killing curves for E. coli and R. prowazeki were not identical, they were similar, indicating that R. prowazeki possess no unusual resistance to killing by superoxide. Rickettsial killing was completely inhibited by the addition of 1mg/ml superoxide dismutase (SOD; Table 5) or allopurinol (APO); SOD and APO completely inhibited the accumulation of detectable superoxide. The addition of lactoperoxidase and KI to this system, however, greatly enhanced killing of rickettsiae. This indicated that rickettsiae were susceptible to normal intraleukocytic oxidative bactericidal mechanisms.

Because these bactericidal mechanisms are known to be generated through stimulation of PMN hexose monophosphate shunt (HMP) activity during phagocytosis (12), we assessed conversion of 1-^{14}C-glucose to $^{14}CO_2$ by PMN in the presence of E. coli or R. prowazeki; some bacteria have been reported to avoid the PMN oxidative bactericidal systems by not stimulating the HMP shunt during phagocytosis (8). Fig 8 shows that PMN HMP shunt activity was stimulated in the presence of either bacterium, although stimulation in the presence of E. coli was somewhat greater.

It was difficult to directly assess intraleukocytic killing of rickettsiae because so few rickettsiae were phagocytized. Alterations in incubation conditions - such as increasing the number of rickettsiae or adding antibody - which might increase the number of ingested rickettsiae, could not be used; PMN were severely damaged by the increased numbers of rickettsiae, and antibody not only blocked the assay system we used (hemolysis) but also has been shown (7) to allow macrophages to be more efficient in killing virulent rickettsiae. Using a multiplicity of 20, however, we were able to demonstrate that only few of the ingested rickettsiae were killed. Fig 8 shows that 45 min after phagocytosis was completed, less than 0.15 $\log_{10}$ rickettsiae were inactivated. Thus, most of the ingested rickettsiae were not inactivated, although we demonstrated that they were susceptible to superoxide and activated halide.

PMN were examined by transmission electron microscopy to determine if ingested rickettsiae remained within phagosomes. Rickettsiae were observed both in phagosomes and free in the cytoplasm. Thus, some rickettsiae are apparently able to circumvent the PMN oxidative bactericidal system by

entering the PMN cytoplasm.

DISCUSSION

Although typhus is not an exudative disease, PMN accumulate locally after rickettsiae burst from the infected endothelium and at least some rickettsiae are phagocytized. It is unlikely, however, that PMN play an important role in the course of typhus as these early PMN:rickettsia interactions fail to protect the nonimmune host. This is probably due, in part, to rickettsial reentry into the endothelium before significant numbers of PMN accumulate. Here, where they are inaccessible to PMN, the rickettsiae multiply slowly as the short-lived PMN (13) are replaced by macrophages (25). Most investigators have turned to the longer lived and more pernicious and versatile macrophages to define rickettsial virulence determinants. Indeed, Gambrill and Wisseman (7) showed that, in the absence of antibody, virulent but not attenuated typhus rickettsiae survived in some macrophages. This, then, suggested that at least some rickettsiae may be unusual in their interactions with other phagocytic host cells, including PMN. We found not only that attenuated typhus rickettsiae were phagocytized poorly by rabbit PMN as compared to E. coli and T4 N. gonorrhoeae, but some ingested rickettsiae were not destroyed and escaped the phagosome into the PMN cytoplasm. This is similar to the report of Ito, et al. (personal communication) that phagocytized R. tsutsugamushi lyse the PMN phagosome and escape into the host cell cytoplasm.

Phagocytosis of rickettsiae ranged from about 2 to 10% of the rickettsial inoculum after 30 min depending on the multiplicity. This was

greater than the 1% or less phagocytosis calculated from the report of Wisseman et al. (23). (These apparent differences may be due to differences in preparation of rickettsiae, source of PMN, viability of rickettsiae, rickettsial strain used, or composition of assay medium.) In both studies, however, the overall rate of phagocytosis was not much different from the rate of internalization of rickettsiae by L cells; approximately 2-4% of an R. prowazeki, Madrid E inoculum was internalized by L cells at 37°C in 30 min. There were, however, fundamental differences between PMN and L cell internalization of rickettsiae. Whereas inactivated ricketsiae were not internalized by L cells (Walker, T. S. and H. H. Winkler. Infect. Immun. 22:200-208, 1978), they were phagocytized by PMN, although not identically to their viable counterparts. This difference between the two systems was because, in the absence of rickettsial energy (and, hence, induced phagocytosis) L cell phagocytosis proceeds very slowly. PMN on the other hand, are "professional phagocytes" and as such are capable of phagocytizing inactivated rickettsiae.

The basis for rickettsial resistance to phagocytosis is not known. There is some temptation to suggest that the toxic activity of rickettsiae against PMN which we described was responsible for resistance to phagocytosis. Certainly the rickettsial leukotoxin (which is probably not a soluble product) was responsible for the loss of PMN phagocytic function and the concomitant plateauing of phagocytosis of rickettsiae. Yet, inactivated rickettsiae and low multiplicities of rickettsiae exhibited no leukotoxic activity but were phagocytized very slowly relative to avirulent E. coli or N. gonorrhoeae. There is, then, some yet unidentified

factor which rendered rickettsiae less susceptible to phagocytosis than were some avirulent gram negative bacteria. Perhaps the shape and size of rickettsiae prevents a physical barrier to phagocytosis; Wisseman et al. (23) reported that two other small avirulent rod-shaped bacteria were similar to rickettsiae in susceptibility to phagocytosis.

Incubation of high multiplicities of rickettsiae with PMN resulted in release of PMN lactate dehydrogenase, formation of free fatty acids and lysophosphatidlethanolamine and loss of PMN phagocytic function. This leukotoxic activity was dependent on rickettsial viability and correlated with rickettsial hemolytic activity; there was a linear relationship between the number of hemolytically active rickettsiae and the extent of damage to PMN, and treatments which inhibited rickettsial hemolysis ($4^{\circ}C$, NaF, inactivation of rickettsiae) also inhibited the formation of lysophosphatides in PMN:rickettsia suspensions. Thus, there is circumstantial evidence that rickettsiae damage PMN by the same process through which they lyse erythrocytes. Consistent with this hypothesis is the observation that rickettsiae damaged PMN "from without"; cytochalasin B, which inhibited phagocytosis, did not inhibit release of LDH and the presence of opsonizing immune serum moderately inhibited LDH release. Yet, at no time did a significant portion of the rickettsiae adhere to the PMN surface. This would seem to indicate that either the leukotoxic activity requires only brief contact between a rickettsia and a PMN or the leukotoxin is a soluble product. It is unlikely, however, that the leukotoxin is a soluble product because incubation of PMN with rickettsial supernatants (alone) or rickettsia:PMN supernatants did not result in PMN

damage (data not shown). We feel that as native rickettsiae collide with PMN, there is a brief period of association during which a phospholipase is activated and some localized hydrolysis of membrane phospholipids occurs. When few rickettsiae are present, the extent of phospholipase activity is not sufficient to significantly damage PMN. Incubation of PMN with large numbers of rickettsiae, however, results in a tremendous escalation of phospholipid hydrolysis concomitant with increase in the number of rickettsial "hits" on each PMN and the PMN membrane is sufficiently damaged to allow leakage of cytoplasmic constituents and loss of phagocytic function. Of course the most obvious question is whether this leukotoxic activity plays a significant role in the course of a normal rickettsial infection. Although there may be circumstances where a large number of rickettsiae will burst from an endothelial cell in the vicinity of an isolated PMN and damage that PMN, it seems more likely that the early PMN response is sufficient to maintain a reasonably low rickettsia:PMN multiplicity and that the rickettsial leukotoxic activity is merely a serendipitous manifestation of the process used by rickettsiae to escape phagosomes after ingestion by a target cell such as an endothelial cell or macrophage. In the case of the endothelial cell, this offers a convenient mode of entry into the host cell cytoplasm where the rickettsiae has ready access to precursors and energy sources for use in self replication. Phagocytosis by a macrophage or a PMN offers additional need for rickettsiae to escape from the phagocytic vacuole as PMN (12) and perhaps macrophages (1) produce superoxide and activated halide which were effective <u>in vitro</u> rickettsiacidal agents. Because PMN HMP shunt activity

was stimulated in the presence of rickettsiae, phagocytosis of rickettsiae should result in their rapid inactivation. Instead, few internalized rickettsiae were inactivated and some rickettsiae were observed free in the PMN cytoplasm. The lytic mechanism then, apparently provide the rickettsia with a means to escape the granulocytic phagosome. Although the long-term benefits of rickettsial survival with PMN are not great because of the short life of and minimal role played by PMN during rickettsial infections, the PMN may serve a model for rickettsial activities within macrophages where survival should result in rickettsial dissemination throughout the body as the macrophage serves as a protective vehicle for such rickettsiae. Although Gambrill and Wisseman (7) reported that some virulent rickettsiae survive within macrophages, it is not known if R. prowazeki escape macrophage phagosomes by the mechanism suggested herein.

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Table 1. Effect of suspending medium on rickettsial hemolytic activity and phagocytosis of E. coli by PMN.

Suspending Medium[a]	Hemolysis[b] (A543) 0 min	Hemolysis[b] (A543) 60 min	Phagocytosis[c] (bacteria x 10^{-8})
SPGMg	0.89	0.70	-
HBG	0.18	0.05	2.6
HBGGlut	0.36	0.37	-
HBGMgGlut	0.64	0.72	3.6

[a] SPG = Sucrose-phosphate-glutamate, HBG = modified Hank's with bovine serum albumin and glutamate added, HBGMgGlut = HBG plus magnesium and glutamate. See text.

[b] Rickettsiae were incubated for 0 and 60 min as indicated at 34°C in the media indicated, and then incubated with sheep erythrocytes for 30 min at 34°C in the same medium as described.

[c] 6.25×10^8 E. coli and 1.25×10^8 PMN were suspended in a 12 x 100 mm tube and tumbled end over end as described.

Table 2. Effects of various treatments on phagocytosis of rickettsiae.

Treatments[a]	Phagocytosis[b]
None	100
4°C (5)	25 ± 0.8
NaF (1)	33
Cytochalasin B (4)	14 ± 2.6
Ab (2)	252 ± 16.5

[a] Rickettsiae and PMN, at a multiplicity of 5, were indicated as described in the presence of the indicated treatments. NaF = 40 mM, Cytochalasin B = 10 µg/ml, Ab = 10% anti-R. prowazeki rabbit immune serum. Number of determinations in parenthesis.

[b] Expressed as percent of 37°C control value ± standard error of the mean.

Table 3. Rickettsial leukotoxic activity.

Incubation Conditions[a]	*E. coli* phagocytized[b] (%)	LDH released[c] (%)
HBGMgGlut (3)	45 ± 4	5 ± 1
R_{NEM} (1)	49	2
R_{native}	15 ± 4	30 ± 3

[a] 1.25×10^8 PMN were incubated with 6.25×10^9 rickettsiae for 20 min at 37°C with tumbling, and then were incubated with 1.25×10^9 *E. coli* for 20 min as described. Number of determinations is indicated in parenthesis.

[b] Expressed as percent of the total *E. coli* inoculum ± the standard error of the mean.

[c] Expressed as percent of total intracellular PMN lactate dehydrogenase (LDH) ± the standard error of the mean.

Table 4. Effect of various treatments on phagocytosis of R. prowazeki by PMN and release of PMN LDH.

Incubation conditions	Rickettsia:PMN multiplicity	PMN-associated rickettsiae ($X10^7$)	Percent Phagocytosis[a]	Percent P LDH relea
R_{native}, 37°C				
Control (9)[c]	50	40	6 ± 1	32 ± 3
(3)	5	12	19 ± 5	5 ± 3
Cytochalasin B (2)	50	14	2 ± 1	30 ± 12
Anti-R Ab (1)	50	245	39	25
(1)	5	23	36	5
NRS (1)	50	51	8	24
R_{NEM}, 37°C				
Control (5)	50	51	8 ± 3	4 ± 4
Cytochalasin B (1)	50	26	4	6
R_{native}, 4°C (1)	50	17	3	7
(1)	5	2	3	6

[a] Percent of rickettsial inoculum ± the standard error of the mean.

[b] Percent of total PMN lactate dehydrogenase ± the standard error of the mean.

[c] Number of determinations.
Anti-R Ab = Anti-R. prowazeki antiserum diluted 1:1 with HBGMgGlut, NRS = normal rabbit serum diluted 1:1 with HBGMgGlut, cytochalasin B = 10 µg/ml cytochalasin B, ND = not determined.

Table 5. Effect of addition of various effectors on inactivation of rickettsiae by the purine-xanthine xoidase system.

Additions [a,b]	Log_{10} rickettsiae inactivated
None	0.57
SOD	0.00
SOD, cat	0.00
SOD, LPO, KI	1.46
APO	0.00

[a] Rickettsiae were incubated with xanthine oxidase (0.5 μ/ml) and purine for 30 min at 34°C in the presence of the indicated additions.

[b] SOD = superoxide dismutase, cat = catalase, LPO = lactoperoxidase, KI = potassium iodide, APO = allopurinol.

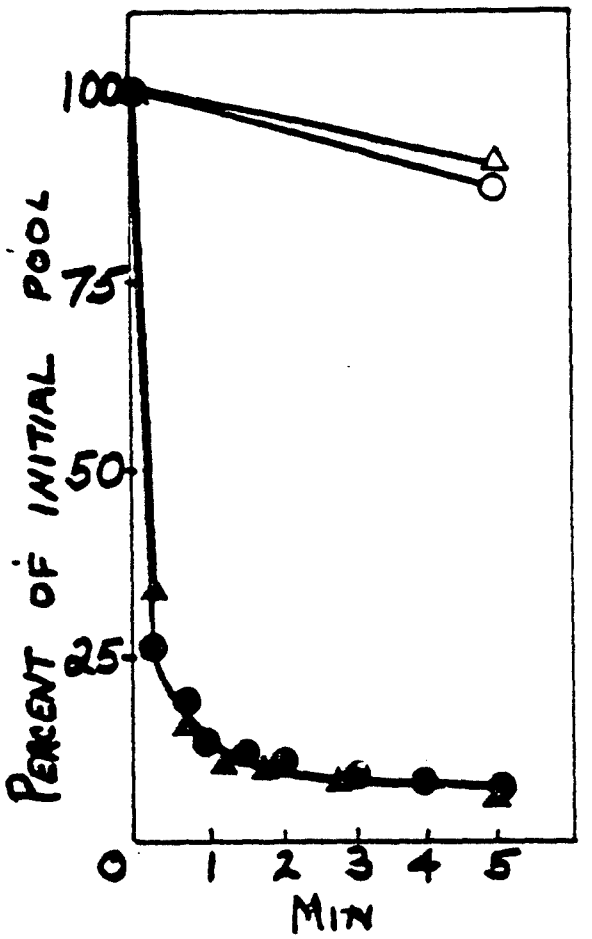

Fig 1. Exit of α-^{32}P-ATP from rickettsiae suspended in SPGMg (▲) or HBGMgGlu (●) before (△,○) and after (▲,●) addition of excess unlabeled ATP.

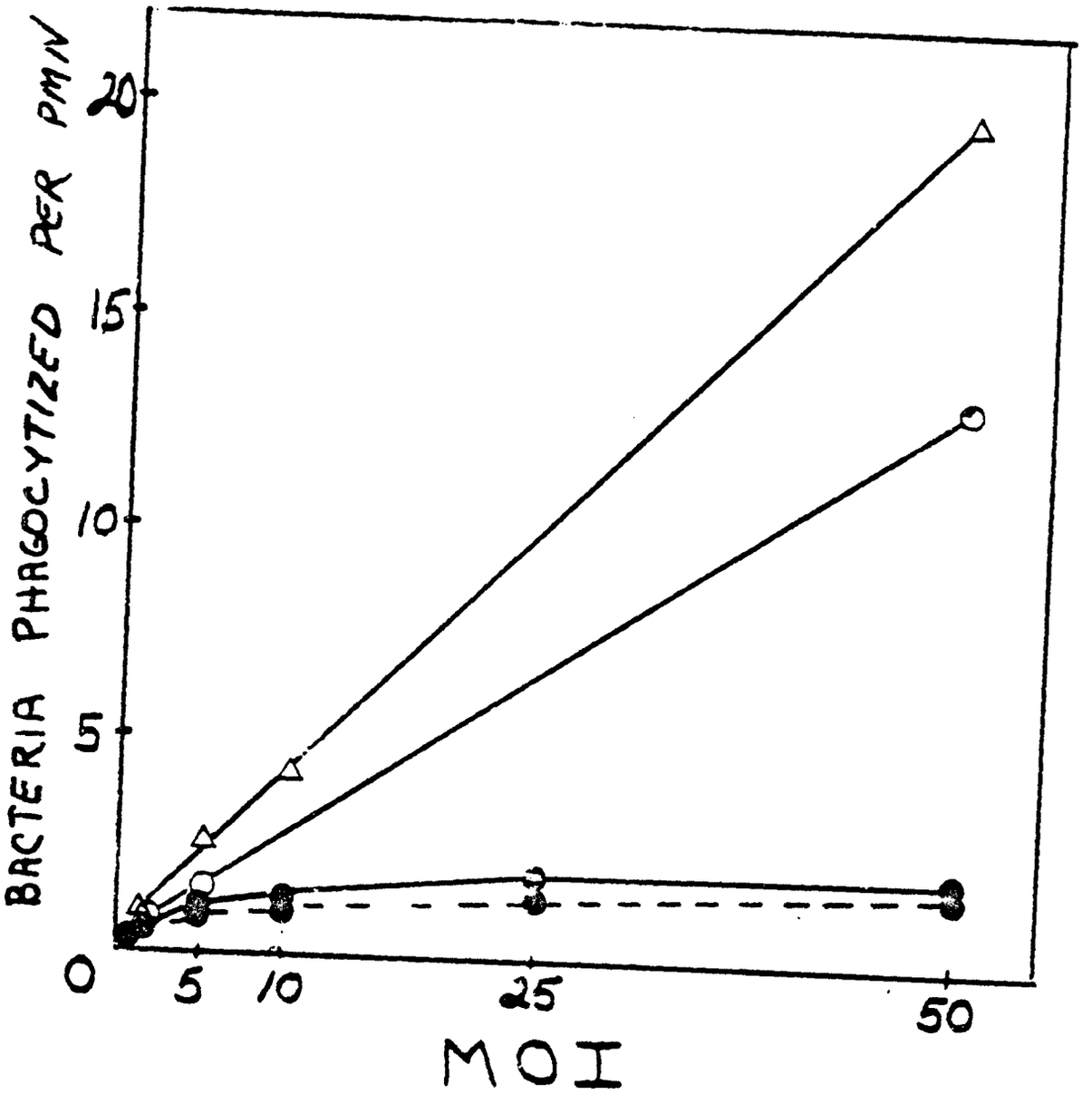

Fig 2. Phagocytosis of rickettsiae (●), E. coli (△) and T4 N. gonorrhoeae (○) by rabbit PMN in vitro. Both total PMN-associated bacteria (solid lines) and phagocytized (dashed line) rickettsiae are delineated.

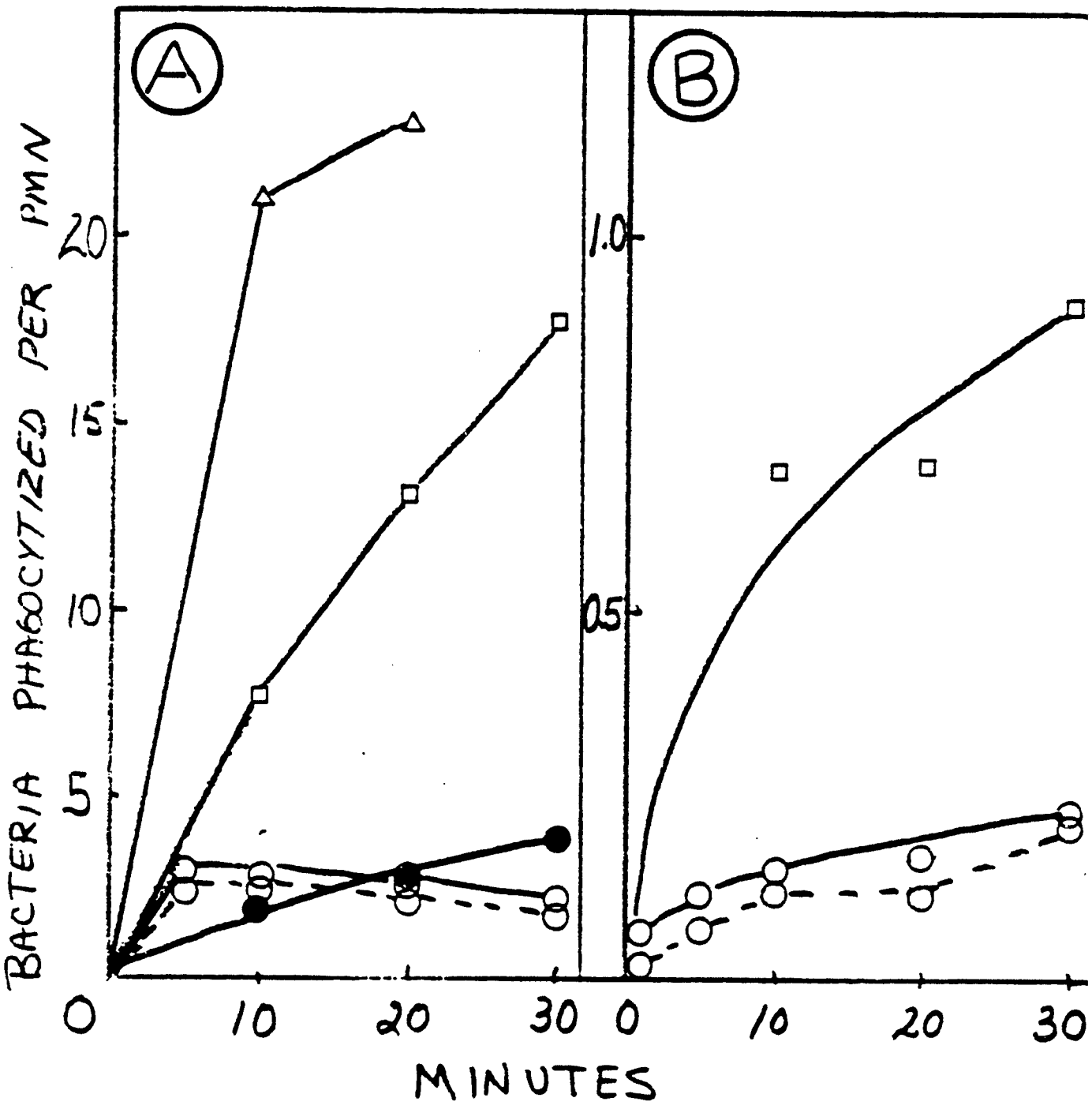

Fig 3. Phagocytosis of native (○) or NEM-inactivated (●) R. prowazeki, E. coli (□) and T4 N. gonorrhoeae (△) at 37°C at multiplicities of 50 (3A) and 1 (3B) as a function of time. The data are presented as bacteria phagocytized per PMN. The solid lines indicate total PMN-associated bacteria and dashed lines indicate internalized rickettsiae.

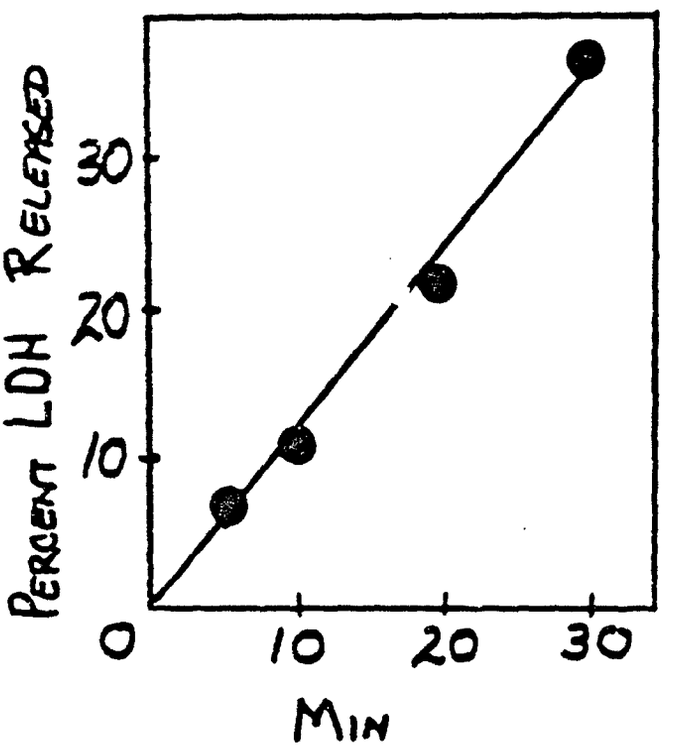

Fig 4. Release of PMN lactate dehydrogenase at 37°C in the presence of native rickettsiae at a multiplicity of 50.

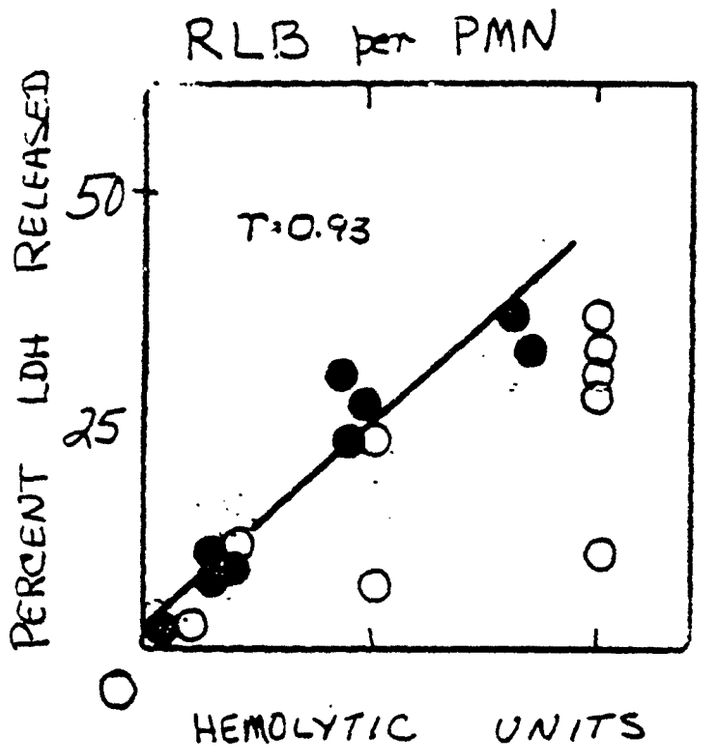

Fig 5. Release of PMN lactate dehydrogenase (LDH) in 30 min as a function of rickettsia:PMN multiplicity (○) or rickettsial hemolytic units per experimental sample (●). r=the linear correlation coefficient for sample He/percent LDH released relationship.

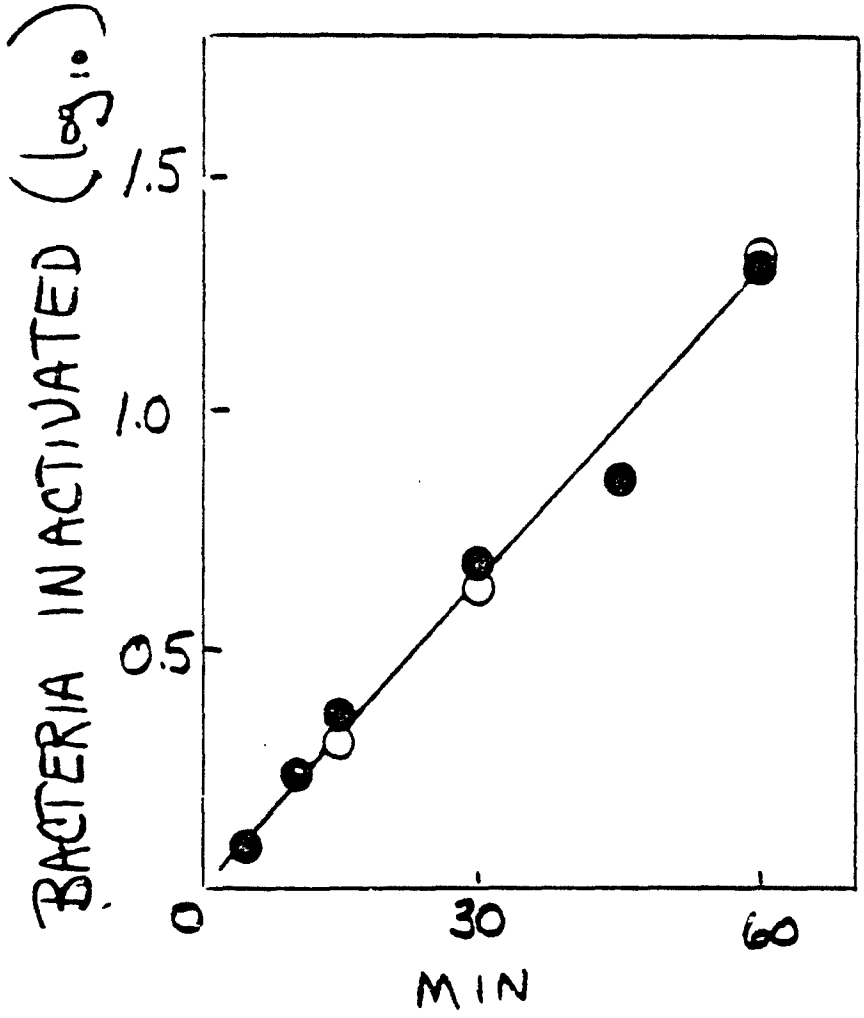

Fig 6. In vitro inactivation of R. prowazeki (●) and E. coli (○) by the xanthine oxidase (XO)-purine system as a function of time. XO concentration was 0.5 u/ml.

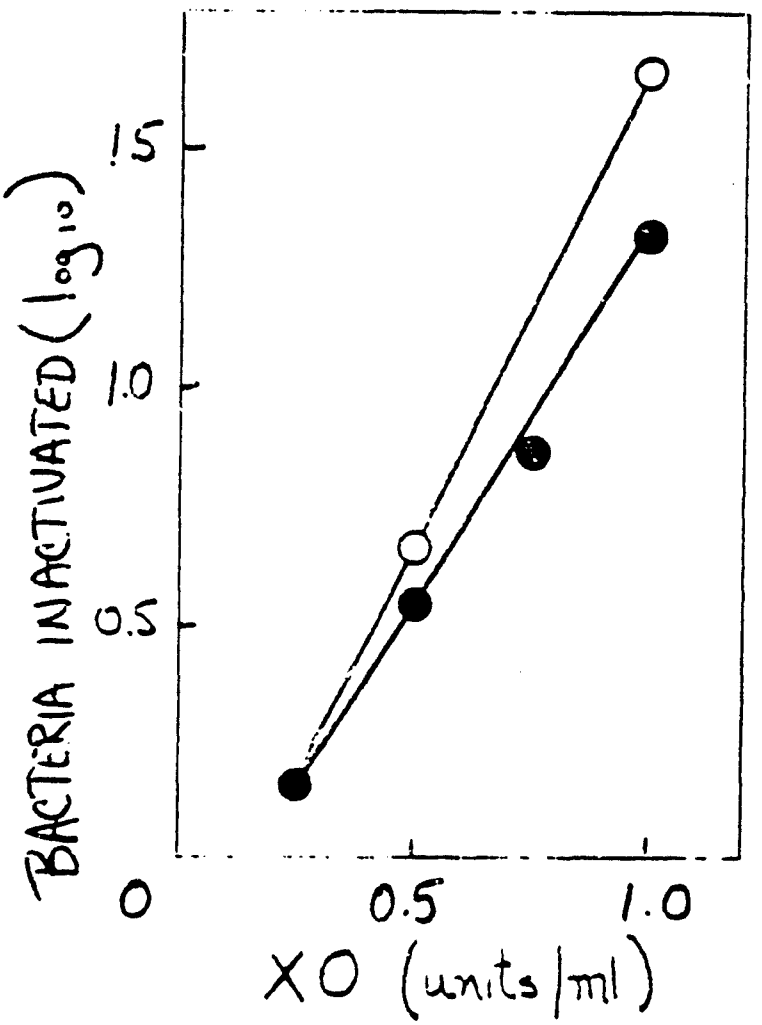

Fig 7. In vitro inactivation of rickettsiae (●) and E. coli (○) by the xanthine oxidase (XO)-purine bactericidal system in 30 min as a function of XO concentration.

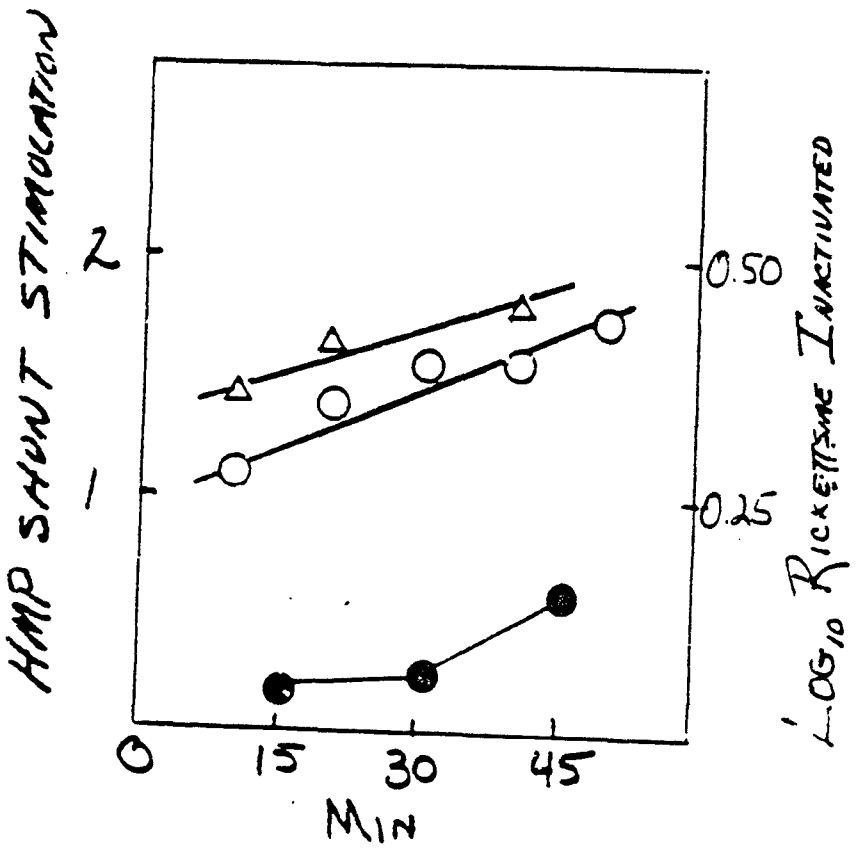

Fig 8. Interactions between PMN and bacteria, Rickettsiae or E. coli were incubated with PMN at a multiplicity of 10 as described. The left axis indicates the $\log_{10}$ phagocytized rickettsiae inactivated (●) whereas the right axis delineates stimulation of 1-^{14}C-glucose oxidation by PMN in the presence of R. prowazeki (○) or E. coli (△) as a function of time.